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A 50-kDa fragment from the NH_2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles

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1. A 50-kDa fragment representing the NH_2-terminus of the heavy subunit of botulinum type A neurotoxin was found, at low pH, to evoke the release of K^+ from lipid vesicles loaded with potassium phosphate. Similar K^+ release was also observed with the intact neurotoxin, its heavy chain and a fragment consisting of the light subunit linked the 50-kDa NH_2-terminal heavy chain fragment. The light subunit alone, however, was inactive.

2. In addition to K^+, the channels formed in lipid bilayers by botulinum neurotoxin type A or the NH_2-terminal heavy chain fragment were found to be large enough to permit the release of NAD (M_r 665).

3. The optimum pH for the release of K^+ was found to be 4.5. Above this value K^+ release rapidly decreased and was undetectable above pH 6.0.

4. The binding of radiolabelled botulinum toxin to a variety of phospholipids was assessed. High levels of toxin binding were only observed to lipid vesicles with an overall negative charge; much weaker binding occurred to lipid vesicles composed of electrically neutral phospholipids.

5. A positive correlation between the efficiency of toxin-binding and the efficiency of K^+ release from lipid vesicles was not observed. Whereas lipid vesicles containing the lipids cardiolipin or dicetyl phosphate bound the highest levels of neurotoxin, the toxin-evoked release of K^+ was low compared to vesicles containing either phosphatidyl glycerol, phosphatidyl serine or phosphatidyl inositol.

6. The implications of these observations to the mechanism by which the toxin molecule is translocated into the nerve ending are discussed.

The neurotoxin of *Clostridium botulinum* type A (BoNT/A) is a 145-kDa protein which acts primarily at the neuromuscular junction causing muscular paralysis by inhibiting the release of the neurotransmitter acetylcholine [1, 2]. The toxin is a two-chain molecule comprising a light subunit (55 kDa) linked by a disulphide bridge to a heavy subunit (95 kDa) [3]. The neuroparalytic activity of BoNT/A is thought to be accomplished in at least three stages: an initial binding stage, an internalisation stage and then one or more steps which disable the acetylcholine release mechanism. BoNT/A has been shown to bind specifically to acceptors on rat brain synaptosomes with high affinity (K_d = 0.6 nM) by an active-site region located on the heavy subunit which is believed to bind the toxin to the presynaptic surface of cholinergic nerve endings prior to internalisation [4, 5]. Little is presently known about the mechanism by which the toxin is internalised. The process is energy-requiring and may resemble the process of receptor-mediated endocytosis [5]. The molecular mechanism by which the toxin inhibits transmitter release is still completely unknown.

Botulinum toxin is structurally similar to both tetanus and diphtheria toxins and for both the latter toxins it has been demonstrated that an NH_2-terminal portion of the larger subunit induces pore formation in lipid bilayers, a property which may be relevant to the transport of an active toxin fragment into the cytoplasm [6–8]. Using planar lipid bilayers, similar pore formation by both botulinum type C_1 neurotoxin [9] and the heavy subunit of botulinum type B neurotoxin has also been demonstrated [10].

In this study, using liposomes of defined phospholipid composition, it is shown that BoNT/A is capable of forming channels in lipid bilayers at low pH. It is further demonstrated that this channel-forming activity is retained by a 50-kDa fragment representing the NH_2-terminal portion of the heavy subunit. The properties of the lipid-toxin interaction are examined and discussed in relation to a possible mechanism for toxin internalisation.

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Abbreviations. BoNT/A, *Clostridium botulinum* type A neurotoxin; mouse LD_{50}, amount of toxin that killed 50% of mice injected (i.p.) with toxin; PtdCho, phosphatidyl choline; PtdGro, phosphatidyl glycerol; PtdEtn, phosphatidyl ethanolamine; PtdIns, phosphatidyl inositol; PtdCho/PtdGro vesicles, liposomes consisting of equimolar proportions of PtdCho and PtdGro.

Enzyme. Trypsin (EC 3.4.21.4).

MATERIALS AND METHODS

Materials

Phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, soybean phosphatidyl choline (type IIS), cardiolipin, dicetyl phosphate and gramicidin were obtained from the Sigma Chemical Company. Phosphatidyl choline (lecithin grade I) and urea (Aristar

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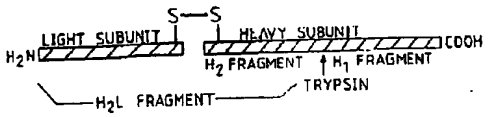

Fig. 1. Clostridium botulinum *type-A neurotoxin*

grade) were supplied by BDH Chemicals Ltd. [*carbonyl*-^{14}C]NAD and [^{125}I]iodine were obtained from Amersham International.

Purification of Clostridium botulinum *type A neurotoxin and its tryptic fragments*

C. botulinum type A neurotoxin was purified to a specific toxicity of $1-2\times10^8$ mouse LD_{50} mg^{-1} by affinity chromatography on *p*-aminophenyl-β-D-thiogalactopyranoside as described previously [11]. The H_2L fragment (see Fig. 1) (specific toxicity $6-12\times10^3$ mouse LD_{50} mg^{-1}) was purified by a modification of a previously described procedure [11]. Botulinum neurotoxin (40 mg in 15 ml) in 0.15 M Tris/HCl buffer, pH 8.0 containing 100 mM NaCl was treated with trypsin (50 μg ml^{-1}) for 72–96 h at 20°C, dialysed against 20 mM triethanolamine buffer, pH 7.8 containing 100 mM NaCl, filtered (0.22 μm pore size) and then chromatographed (fast protein liquid chromatography system, Pharmacia) on a Mono Q column (HR 10/10, Pharmacia) equilibrated in the latter buffer. The column was washed with a further 100 ml of the triethanolamine buffer and then the H_2L fragment eluted with triethanolamine buffer (20 mM, pH 7.8) containing 200 mM NaCl. The purified fragment (≈ 1.5 mg ml^{-1}) was made 5 μg ml^{-1} with trypsin inhibitor, dialysed against 0.15 M Tris/HCl buffer, pH 8.0 containing 50 mM NaCl and stored at −20°C. The heavy chain and H_2 fragment of botulinum type A neurotoxin were purified from the neurotoxin and H_2L fragment respectively by precipitating the light subunit in the presence of 2.5 M urea, 1 M NaCl and 100 mM dithiothreitol as described previously [11]. The light subunit of botulinum type A neurotoxin (< 100 mouse LD_{50} mg^{-1}) was purified from the H_2L fragment by the method of Kozaki et al. [12].

Preparation of liposomes and K^+ release studies

Lipid vesicles were prepared essentially by the method of Enoch and Strittmatter [13]. Phospholipid mixtures (60 μmol total lipid) were suspended in 3 ml 0.1 M potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and 10 mM sodium deoxycholate and then briefly sonicated (three 5-s periods, Dawe Instrument type 7530 A, setting 3) in ice under nitrogen gas. Sodium deoxycholate was removed from the liposomes by dialysis three times against 500 ml 0.1 M potassium phosphate buffer, pH 7.2 containing 1 mM EDTA at 4°C. Soybean phospholipids were first washed with acetone prior to liposome preparation [14]. Liposomes made with 0.1 M potassium acetate buffer pH 4.5 were prepared by the freeze-thaw technique of Kasahara and Hinkle [14].

Detection of K^+ release from lipid vesicles was carried out essentially as described by Boquet and Duflot [8]. Lipid vesicles (50 μl) were added to 15 ml of the appropriate buffer and slowly stirred. Potassium release was monitored using a Philips K^+ electrode connected via Philips model 9421 pH meter to an Oxford Instruments 3000 chart recorder.

Release of [^{14}C]NAD from liposomes

For studying the release of [^{14}C]NAD, liposomes were prepared as above with 3 ml 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mM EDTA and [^{14}C]NAD (37 kBq ml^{-1}) using half the concentration (5 mM) of sodium deoxycholate. The detergent was then removed by gel filtration on a column of Sephadex G-50 (25 × 3 cm) equilibrated in potassium phosphate buffer pH 7.2 containing 1 mM EDTA. Liposomes, eluted in the void volume, were then dialysed against 1 l of the latter phosphate buffer. Release of NAD was assessed with 1.5-ml portions of liposome preparations. After diluting to 11.5 ml with 0.1 M sodium acetate buffer, pH 4.3 (to give a final pH of 4.5) either buffer, toxin of Triton X-100 was added and the mixture incubated for 30 min at 20°C before being centrifuged at 200000 × *g* for 1 h. Portions (1 ml) of the supernatant fluids were removed and their radioactivity assessed by scintillation counting.

Binding of radioiodinated botulinum neurotoxin to liposomes

Botulinum neurotoxin, radioiodinated using chloramine-T [4], was mixed with unlabelled toxin to give a specific activity of 1.7 MBq mg^{-1} and further diluted with 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mg ml^{-1} ovalbumin to a neurotoxin concentration of 100 μg (0.17 MBq) ml^{-1}. Liposomes (0.5 ml) prepared as described above were mixed with 11 ml 0.1 M sodium acetate buffer, pH 4.4 and 100 μl of the radiolabelled toxin solution, incubated for 30 min at 20°C and then centrifuged at 200000 × *g* for 1 h. The supernatant fluid was carefully removed and the excess fluid allowed to drain from the liposome pellet by inverting the tubes for 5 min. The liposomes were resuspended in 1 ml 0.1 M sodium phosphate buffer, pH 7.2 and the radioactivity in 100-μl portions measured.

RESULTS

Release of K^+ from phosphatidyl choline/phosphatidyl glycerol vesicles

Addition of 10 μg of the channel-forming polypeptide gramicidin to phosphatidyl choline/phosphatidyl glycerol (molar ratio, 1:1) liposomes (PtdCho/PtdGro vesicles) loaded with K^+ caused a rapid (< 30 s) releases of K^+ at pH 4.0 (Fig. 2a). No further release of K^+ was detected when an additional 10 μg gramicidin was added, indicating that all the available K^+ had been released. An equivalent quantity of K^+ was liberated at pH 7.2 showing that the low pH had not affected the integrity of the vesicles.

Addition of increasing amounts of the H_2 fragment of botulinum type A neurotoxin to PtdCho/PtdGro vesicles suspended in buffer at pH 4.0 evoked the release of K^+ ions (Fig. 2b and c). Under the experimental conditions, the addition of 1 nmol of the H_2 fragment released all of the available K^+ in less than 1 min; addition of gramicidin at this point caused no further release of K^+. Treatment of the vesicles with a further portion of the H_2 fragment after gramicidin treatment had no effect of the baseline value. At pH 7.2 the H_2 fragment was completely ineffective at releasing K^+ from vesicles (Fig. 2d).

Analogous experiments conducted with the intact neurotoxin, heavy chain and H_2L fragment of BoNT/A yielded qualitatively similar results. The results for the intact toxin are shown in Fig. 3a–c. At pH 4.0, BoNT/A, the H

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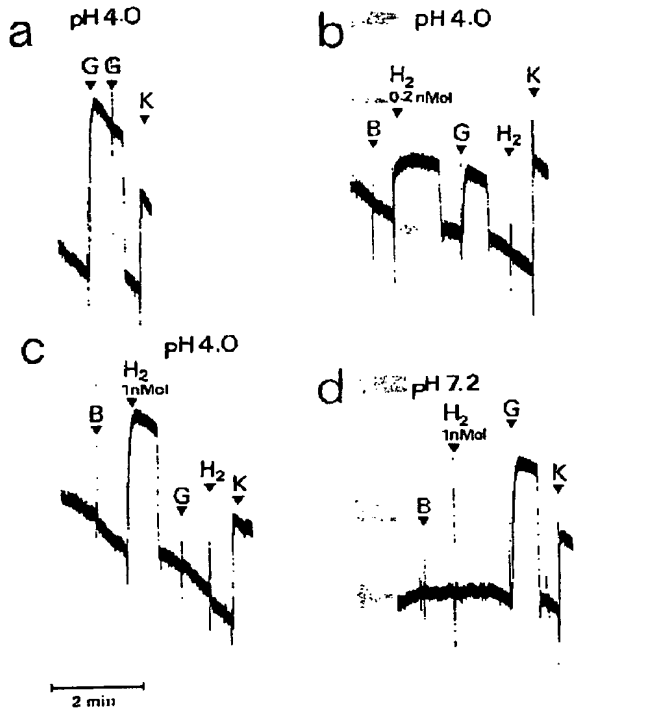

Fig. 2. *Release of K^+ from PtdCho/PtdGro vesicles by the H_2 fragment of BoNT/A.* (a) After addition of vesicles to 0.1 M sodium acetate buffer, pH 4.0, gramicidin (G, 10 µl of 1 mg ml^{-1} solution in ethanol) was added. When maximal response had been obtained, the baseline was adjusted to zero and KCl (K, 10 µl of a 20 mM KCl solution) was added as a standard. (b, c) After the addition of the fragment buffer as a control (B), the H_2 fragment was added to vesicles in 0.1 M sodium acetate buffer, pH 4.0, at the indicated amounts. At maximal response, the baseline was returned to zero and gramicidin added as above (G) followed by baseline adjustment and further addition of the same amount of the H_2 fragment. Finally KCl standard was added as in (a). (d) The H_2 fragment was added to vesicles in 0.1 M sodium phosphate buffer, pH 7.2; additions of buffer (B), gramicidin (G) and KCl (K) were as described in (b). The volume of the reaction mixture in each case was 15 ml

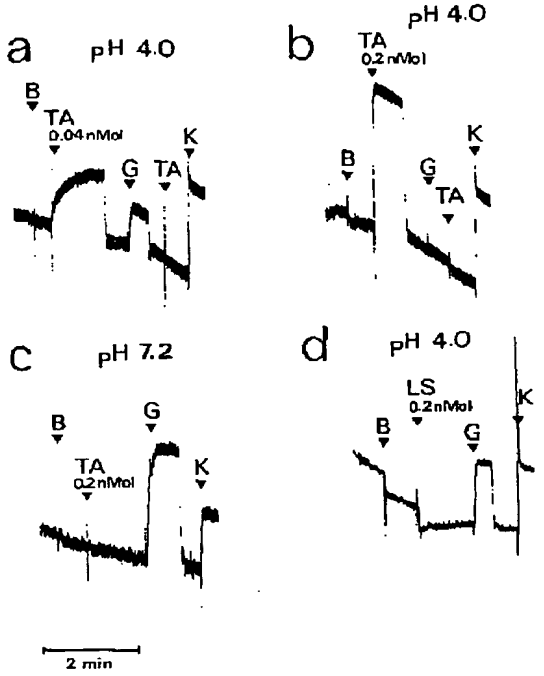

Fig. 3. *Release of K^+ from PtdCho/PtdGro vesicles by BoNT/A and its light subunit.* (a, b) BoNT/A (TA) was added to vesicles in 0.1 M acetate buffer, pH 4.0, at the indicated amounts. (c) BoNT/A was added to vesicles in 0.1 M sodium phosphate buffer, pH 7.2. (d) Light subunit (LS) was added to vesicles in acetate buffer pH 4 at the indicated amount. Baseline adjustments and additions of the fragment buffer control (B), gramicidin (G) and KCl (K) were as described in Fig. 2

fragment and the heavy chain were found to be more efficient at releasing K^+ from PtdCho/PtdGro vesicles than the H_2 fragment; on a molar basis 4–5-fold less of the heavy chain, H_2L fragment and intact toxin consistently gave the same release of K^+ as the H_2 fragment. At pH 4.5, however, H_2 fragment was equally as effective at releasing K^+ from lipid vesicles as the intact neurotoxin (see below). As found for the H_2 fragment, at pH 7.2 intact toxin (Fig. 3c) heavy subunit and H_2L fragment did not release K^+ from lipid vesicles.

The light subunit of BoNT/A was ineffective at releasing K^+ from lipid vesicles at both pH 4.0 (Fig. 3d) and pH 4.5. BoNT/A which had been inactivated with formaldehyde retained less than 3% of the K^+-releasing activity of the native neurotoxin.

Similar results were obtained when crude soybean phospholipid vesicles were substituted for the PtdCho/PtdGro vesicles. The amount of the neurotoxin and its fragments required to release all the K^+ from soybean lipid vesicles, however, had to be increased by 3–5-fold over that required for PtdCho/PtdGro vesicles.

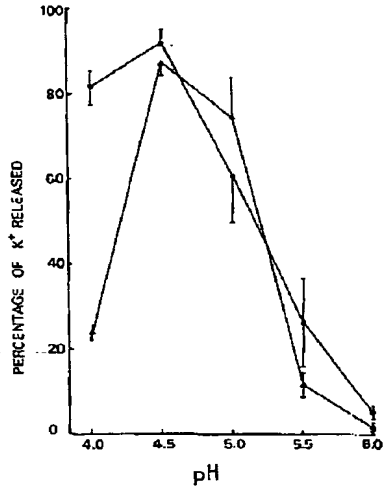

Fig. 4. *Effect of pH on the release of K^+ from PtdCho/PtdGro vesicles by BoNT/A and its H_2 fragment.* Liposomes (50 µl) were suspended in 15 ml 0.1 M sodium citrate/sodium phosphate buffer at each pH and 0.2 nmol of either BoNT/A (●) or the H_2 fragment (▲) added. The amount of K^+ released was assessed by measuring the response 80 s after addition of the toxin and expressing this value as a percentage of the response obtained with the gramicidin control at the same pH value. Values represent the mean of at least five measurements

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Table 1. *Release of [^{14}C]NAD from PtdCho/PtdGro vesicles by BoNT/A or its H_2 fragment at pH 4.5*
The amount of NAD released after disruption of the liposomes with Triton X-100 was taken as 100% release

Treatment of liposomes	Total ^{14}C in the supernatant fluid		Average NAD released
	1	2	
	cpm		%
Control (no addition)	6520	7394	49.6
Intact BoNT/A (2 nmol total)	11994	12914	88.7
H_2 fragment (2 nmol total)	10764	11741	80.2
Triton X-100 (1% final concn)	13328	14731	100

Table 2. *Binding of ^{125}I-BoNT/A to, and release of K^+ from liposomes of different phospholipid composition*
For the assessment of BoNT/A binding each incubation mixture (11.5 ml) contained 10 μg neurotoxin (17 kBq) and 10 μmol lipid. The percentage of K^+ released by 0.2 nmol BoNT/A from liposomes in 0.1 M sodium acetate buffer, pH 4.5 was assessed as described in Fig. 4

Phospholipid composition of liposomes (molar ratio)	^{125}I-BoNT/A bound	K^+ released
	% total	%
PtdCho/PtdGro (1:1)	51 ± 5.0	> 95
Soybean	71 ± 4.5	63 ± 16
PtdCho	4.0 ± 0.6	< 5
PtdCho/PtdEtn (1:1)	10.5 ± 4.0	< 5
PtdCho/PtdIns (1:1)	65 ± 2.4	> 95
PtdCho/PtdSer (1:1)	46 ± 7.0	> 95
PtdCho/cardiolipin (2:1)	80.5 ± 9.7	31 ± 6.6
PtdCho/dicetylphosphate (2:1)	90 ± 4.3	< 5
Negative control	3.3 ± 1.8	—

Effect of pH on the release of K^+ from lipid vesicles

At extra-vesicular pH values between 4.5 and 6.0, the extent to which K^+ was released from PtdCho/PtdGro vesicles was similar for both the intact botulinum neurotoxin and its H_2 fragment. Maximal release occurred at pH 4.5 (Fig. 4): above pH 4.5 the K^+ release rapidly decreased and was undetectable above pH 6.0. At pH 4.0, however, while the K^+ release by the intact neurotoxin was only slightly lower than that observed at pH 4.5, an almost fourfold reduction in K^+ release by the H_2 fragment was observed.

Changes in the pH value of the intra-vesicular environment did not appear to affect significantly the release of K^+ from lipid vesicles. Liposomes containing 0.1 M potassium acetate buffer at pH 4.5 instead of potassium phosphate buffer at pH 7.2 were found to release similar amounts of K^+ when the extravesicular environment was reduced to pH 4.5 in the presence of botulinum toxin.

Release of [^{14}C]NAD from PtdCho/PtdGro vesicles

At pH 4.5, addition of either BoNT/A or the H_2 fragment to PtdCho/PtdGro vesicles loaded with [^{14}C]NAD released over 80% of the total radioactivity (Table 1). Values obtained for the release of NAD in the presence of the neurotoxin consistently were close to double those of control values obtained in the absence of toxin, indicating that the membrane channels formed by BoNT/A are large enough to permit the release of NAD molecules (M_r 665).

Binding of ^{125}I-BoNT/A to and release of K^+ from liposomes of different phospholipid composition

At pH 4.5, the highest levels of ^{125}I-labelled BoNT/A binding were observed to liposomes comprising phospholipids with a net negative charge; liposomes incorporating either PtdGro, PtdIns or PtdSer bound approximately 50% of the total toxin and those including either dicetyl phosphate or cardiolipin, over 80% of the total toxin (Table 2). In contrast, much lower toxin binding was observed to liposomes made with the neutral phospholipids PtdCho and PtdEtn. At pH 7.2, however, less than 7% of the radiolabelled toxin was recovered in the liposome pellet regardless of the phospholipid composition. The binding of botulinum toxin to PtdCho/PtdGro vesicles was found to be partially reversible in that 45% of the toxin bound to the liposomes at pH 4.5 could be released when the pH was raised to 7.2. Iodination of BoNT/A did not affect the pore-forming properties of the toxin. Neurotoxin iodinated with increased concentrations of iodine, resulting in approximately 4 mol iodine/mol toxin, still retained more than 90% of the channel-forming ability of the untreated controls.

Whereas low levels of toxin binding to liposomes were associated with poor release of K^+ from the lipid vesicles, high levels of binding were not always accompanied by efficient K^+ release. Thus, although lipid vesicles prepared from either dicetyl phosphate or cardiolipin appeared to display the highest affinity for the neurotoxin, toxin-associated release of K^+ from these vesicles was low compared to that from vesicles comprising either PtdGro, PtdSer or PtdIns. Similarly, compared to lipid vesicles containing the latter three phospholipids, the toxin-induced release of K^+ from soybean phospholipid vesicles was significantly lower even though all these liposome preparations bound comparable amounts of toxin (Table 2).

DISCUSSION

The molecular details of the events leading to internalisation of botulinum toxin (or an active toxin fragment) into the cytoplasm of presynaptic nerve cells are only vaguely understood. Acceptor molecules present on the presynaptic nerve surface bind tightly to the molecules of neurotoxin which then cross the outer plasmalemma by an energy-dependent process resembling receptor-mediated endocytosis [5]. The mechanism by which the toxin penetrates the lipid bilayer, whether it be the membrane of the plasmalemma or an endosomal vesicle, however, is still unresolved. Lipid bilayers in the form of liposomes provide a convenient model for studying these membrane-toxin interactions. In the present study we show that a 50-kDa NH_2-terminal fragment (H_2) of BoNT/A is able to form channels in unilamellar vesicles at

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low pH which are large enough to allow the release of K^+ and NAD^+. Similar channel-forming activity was also observed with the intact neurotoxin, its heavy subunit and H_2L fragment. This property of the neurotoxin appears to be confined to the heavy subunit since no channel-forming activity was evident in purified samples of the neurotoxin light subunit. In addition, the similarity observed between the pore-forming activities of the heavy subunit and the H_2L fragment suggests that H_1 fragment of the neurotoxin has little or no channel-forming activity compared to the H_2 fragment.

The pH optimum of 4.5 for membrane channel formation observed for type A neurotoxin is close to values obtained in similar studies with diphtheria toxin [7] and tetanus [8] and botulinum type B neurotoxins [10]. Botulinum type C_1 neurotoxin, however, has recently been reported to form channels in planar lipid bilayers at a higher pH optimum of 6.1 [9]. One explanation for the low pH requirement of type A neurotoxin for membrane channel formation is that as the pH is lowered a hydrophobic site is exposed on the H_2 component which facilitates membrane binding. Supportive of this proposal is the observation that radiolabelled botulinum neurotoxin binds strongly to PtdCho/PtdGro vesicles only at low pH. The exposure of a hydrophobic region, induced by protein conformation changes at low pH, has also been proposed to explain the pH-dependent insertion of diphtheria [15, 16] and tetanus toxin [8] into lipid bilayers. Whatever the mechanism of pore formation for BoNT/A, a pH gradient across the membrane does not appear to be required since reducing the intra-liposomal pH from 7.2 to 4.5 did not affect the toxin-induced release of K^+.

Radiolabelled botulinum type A neurotoxin, at low pH, bound to a variety of liposomal phospholipids. High levels of toxin binding, however, were observed only to liposomes comprising phospholipids with an overall negative charge; liposomes consisting of electrically neutral lipids displayed a much weaker interaction with the toxin. The binding of neurotoxin to lipid vesicle membranes was not always accompanied by the formation of membrane channels. While the toxin bound strongly to lipid vesicles consisting of either soybean phospholipids, cardiolipin or dicetyl phosphate, the release of K^+ from these vesicles was significantly less than that observed from liposomes consisting of either PtdGro, PtdIns or PtdSer which appeared to bind the toxin less strongly. These observations may indicate that toxin binding and channel formation are two distinct events in which case some phospholipids, while allowing the toxin molecule to bind, may inhibit the subsequent channel-forming stage. A similar mechanism has been suggested for botulinum type C_1 neurotoxin for which it has been suggested that aggregates of the toxin may be involved in channel formation [9]. Alternatively, it could be argued that the neurotoxin is able to bind the lipid bilayer in several configurations, not all of which are conducive to the formation of a membrane channel. Whether the toxin binds in a channel-forming configuration or not could, then, be influenced by the nature of the membrane phospholipids. The lack of a positive correlation between toxin binding and pore formation suggests that a specific toxin conformation is required before membrane channels can form and argues strongly against the possibility that the release of K^+ from liposomes is simply due to a non-specific disruption of the integrity of the membrane surface as the toxin binds. The inability of formalin-inactivated toxin to release K^+ from liposomes and the reduced channel-forming activity of the H_2 fragment at pH 4.0 also support the view that there is a requirement for a specific toxin conformation to enable the formation of membrane pores.

Whether or not the channel-forming activity observed for the H_2 fragment of BoNT/A plays a significant role in the neuroparalytic activity of the neurotoxin is uncertain. The ability to form membrane pores at low pH has been found to be a common characteristic of a number of bacterial toxins; in addition to the other botulinum neurotoxins so far studied (types B [10] and C_1 [9]), similar channel-forming activities have been demonstrated in fragments analogous to the H_2 fragment of BoNT/A in both tetanus [8] and diphtheria [7] toxins. In the case of diphtheria toxin direct evidence has recently been obtained that illustrates the importance of a low extravesicular pH in the translocation of the enzymically active fragment A across lipid bilayers [17]. Using liposomes containing the toxin substrates NAD^+ and elongation factor 2, it was shown that only at low extravesicular pH and using intact diphtheria toxin could the enzymically active fragment A enter the liposomes and catalyse the ADP-ribosylation reaction. It is tempting to speculate that a similar translocation mechanism could operate for botulinum toxin. If, as has been suggested [5], BoNT/A initially enters the nerve by a process similar to receptor-mediated endocytosis, then the acidic environment which develops in the endosome [18] should induce the toxin to insert itself in the lipid bilayer. Whether or not the toxin or a toxin fragment subsequently enters the cytosol has yet to be determined. Recent studies may favour the translocation of the whole toxin since intact BoNT/A intracellularly injected into chromaffin cells strongly inhibits secretion [19]. Substantiation of such an internalisation mechanism would imply that the heavy subunit of BoNT/A plays a role in at least two stages in the action of the neurotoxin: firstly, in the binding of the toxin to the presynaptic nerve surface, most probably by an active site region located on the H_1 fragment [11], and secondly, in the translocation of the toxin or a fragment into the cytosol mediated by the H_2 component.

Clearly, in the light of the present findings and those with other bacterial toxins the interaction of botulinum neurotoxin with lipid bilayers at low pH is worthy of continued investigation.

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